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ANGIOSTATIC MECHANISMS OF ENDOGENOUS ANGIOGENESIS INHIBITORS

Niina Veitonmäki

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Front Cover: Apoptotic blood vessels in K1-5-treated tumors

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“... it seems quite possible that the primary difference between the malignant cell and the normal cell from which it arose may be that the malignant cell is able to provoke a continued vascular proliferation.” (Algæ et al, 1945)
LIST OF PUBLICATIONS


* These two authors contributed equally
ABSTRACT

Angiogenesis, the process of sprouting of new capillaries from the existing blood vessels, is essential for tumor growth and metastasis. Angiostatin was discovered as the first specific endogenous angiogenesis inhibitor nearly 10 years ago. The structure of angiostatin consists of the first three/four kringle (K1-3/K1-4) domains of plasminogen. However, plasminogen contains five kringle structures and it has been shown that kringle 5 (K5) displays at least as potent inhibitory activity as angiostatin in suppression of endothelial cell growth. This thesis work describes the discovery of kringle 1-5 (K1-5) as one of the most potent plasminogen-derived angiogenesis inhibitors. It specifically inhibits endothelial cell growth in vitro and angiogenesis in vivo. In a mouse tumor model, K1-5 potently inhibits primary tumor growth at the dose of which angiostatin is inactive. Further mechanistic studies show that K1-5 specifically induces endothelial apoptosis by binding to endothelial cell surface $F_0F_1$-ATP synthase, and activating caspases-3, -8, and -9. In vivo experiments show that endothelial apoptosis is essential for the angiostatic activity of K1-5. In addition to K1-5, we found that adiponectin, a secreted adipocytokine, potently inhibits angiogenesis and tumor growth. Similar to K1-5, adiponectin-induced endothelial apoptosis involves activation of pro-caspases. Thus, our findings demonstrate that induction of endothelial apoptosis is a common pathway for these endogenous inhibitors to specifically inhibit angiogenesis. In the effort of standardizing our in vitro assay systems, primary bovine capillary endothelial (BCE) cells were immortalized using human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase nuclear ribonucleoprotein complex. Surprisingly we have found that the average telomere lengths in the hTERT-transfected cells are shorter than those found in primary pre-senescent cells, indicating that immortalization of these bovine endothelial cells must be mediated by alternative mechanisms. Our results indicate that one of the alternative mechanisms might include inactivation of p16 by hTERT-induced DNA methylation. This thesis work provides important information for developing therapeutic antiangiogenic agents in the treatment of angiogenesis-dependent diseases such as cancer and metastasis.

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LIST OF ABBREVIATIONS

ATP synthase  adenosine triphosphate synthase
BCE cells  bovine capillary endothelial cells
CAM  chorioallantoic membrane
CytC  cytochrome C
DNMT  DNA methyl transferase
ERK  extracellular signal-regulated kinase
EC  endothelial cell
ELISA  enzyme-linked immunosorbent assay
FGF  fibroblast growth factor
HGF  hepatocyte growth factor
HUVEC  human umbilical vein endothelial cells
INK 4A  inhibitor of cyclin dependent kinase 4 and 6
K1-5  kringle 1-5
MAPK  mitogen-activated protein kinase
MMP  matrix metalloproteinase
PDLs  population doublings
pRb  retinoblastoma protein
Pgn  plasminogen
SA–β-gal  senescence associated beta-galactosidase
SMC  smooth muscle cell
t-PA  tissue-type plasminogen activator
TERT  telomerase reverse transcriptase
TNFR1  tumor necrosis factor receptor 1
TRAP  telomeric repeat amplification protocol
TUNEL  terminal deoxynuclotidyl transferase-mediated dUTP nick end labelling
u-PA  urokinase-type plasminogen activator
VEGF  vascular endothelial growth factor
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Normal tissue function depends on an adequate supply of oxygen and nutrients from blood vessels. The observation that angiogenesis occurs in growing tumors was already made nearly 150 years ago (Thiersch, 1865; Goldman, 1907; Algire and Chalkley, 1945). In 1945, Algire and co-workers postulated in that “the rapid growth of tumor transplants is dependent upon the development of a rich vascular supply, and that an outstanding characteristic of the tumor cells is its capacity to elicit continuously the growth of new capillary endothelium from the host”. The research foundation within the field of tumor angiogenesis was laid out by Dr. Folkman in 1971. He suggested that tumors cannot grow beyond the volume of a few cubic millimeters without inducing new blood vessel growth, and that blocking of angiogenesis could be a strategy to halt the tumor growth (Folkman, 1971). The knowledge that many pathological conditions require new blood vessels, and the discovery of factors that mediate angiogenesis has significantly increased our understanding of several pathological processes. This new understanding has opened the way to innovative and fresh approaches to the diagnosis and treatment of angiogenesis dependent diseases, such as tumor growth, metastases, diabetic retinopathy and rheumatoid arthritis.

1.1 VASCULAR DEVELOPMENT

In the embryo, organs and tissues can be vascularized by two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* formation of blood vessels from differentiating endothelial precursor cells, angioblasts, while angiogenesis is the growth of new blood vessels from pre-existing vessels. Further, neovascularization can occur through arteriogenesis, e.g. collateral growth, where collaterals increase lumen volume by proliferation of endothelial- and supporting cells (Carmeliet, 2000). The circulatory system also consists of a secondary component: the lymphatic system. The lymphatic system is a vascular network of thin-walled capillaries and larger vessels lined with a single layer of endothelial cells anchored by filaments into the surrounding tissue. The lymphatic endothelial cells have a valve-like arrangement that drain fluid from the tissue spaces into the vessels, and block reverse
Angiostatic mechanisms of endogenous angiogenesis inhibitors

transport. The lymphatic system collects extravasated proteins and fluid, and returns it to the venous system for recirculation (reviewed in Jussila and Alitalo, 2002).

1.1.1 Vasculogenesis

Angioblasts are endothelial progenitor cells, which have the potency to differentiate into endothelial cells but have not yet acquired all the characteristic markers nor a lumen (Risau, 1995; Risau and Flamme, 1995). In the yolk sac, mesenchyme angioblasts and hematopoetic precursor cells differentiate to form blood islands. The subsequent process of blood island fusion and formation of lumina give rise to a primary capillary plexus. This formation of blood vessels from in situ differentiating endothelial cells is called vasculogenesis. Initially, it was postulated that vasculogenesis occurs only in the developing embryo but recent reports suggest that circulating endothelial precursors can contribute to neovascularization also in the adult (Asahara et al., 1997; Takahashi et al., 1999; Peichev et al., 2000).

1.1.2 Angiogenesis

Angiogenesis, the sprouting or splitting of new capillaries from pre-existing vessels, further refines the primary vascular network and is the principal process by which certain organs, such as the brain, become vascularized. In sprouting angiogenesis, the initiation phase starts with increased vascular permeability followed by a local degradation of the basement membrane and extracellular matrix of the vessel, usually a postcapillary venule as well as the interstitial matrix. The endothelial cells invade the stroma, migrate towards the stimulus and proliferate behind the leading edge. The sprout forms a lumen and reconstructs the basement membrane, and the newly formed vessels are stabilized by recruiting supporting pericytes or smooth muscle cells (Holash et al., 1999). In intussusceptive microvascular growth, a vascular network is formed by insertion of interstitial tissue columns, called tissue pillars or posts, into the vascular lumen. The growth of these columns results in partition of the vessel lumen (Patan et al., 1996).
In healthy adult people, angiogenesis is a rare event. The endothelial cells lining the blood vessel wall are normally quiescent, and the turnover time is hundreds of days. In response to appropriate stimuli, the quiescent vasculature can become activated to induce growth of new capillaries. In adults, angiogenesis is essential for the female reproductive cycle, repair and regeneration of tissues, organ formation, and during wound healing (Folkman, 1995a; Drixler et al., 2002). Regulation and remodeling of fat and tissues have also been reported to be angiogenesis dependent (Folkman, 1998; Franck-Lissbrant et al., 1998; Rupnick et al., 2002).

1.2 ANGIOGENESIS IN DISEASES

Besides the crucial physiological role of angiogenesis in embryogenesis and wound healing, many severe diseases are associated with excessive capillary growth. In
rheumatoid arthritis, new vessels invade the joint surfaces and degrade the cartilage by proteolysis. Intraocular neovascularization, seen in diabetic retinopathy and retinopathy of prematurity, is associated with leaky blood vessels that can easily rupture, which can lead to blindness. Angiogenesis is also known to be involved in psoriasis, infantile hemangiomas and obesity (Folkman, 1995b; Folkman, 1995a; Carmeliet and Jain, 2000). Most importantly, angiogenesis is associated with tumor growth and metastasis. Tumors do not usually grow beyond a volume of a few mm$^3$ unless they become vascularized (Folkman, 1971) The most simple and convincing evidence for this is an experiment where a tiny piece of tumor was implanted in the rabbit cornea (Gimbrone et al., 1974). Following vascularization, the tumor implant grew exponentially and reached beyond the size of the entire eye organ within a few days. However, mechanical disruption of the newly formed blood vessels could totally arrest the tumor growth.

1.3 TUMOR ANGIOGENESIS

There is a general agreement that blood vessels in tumors are structurally and functionally abnormal. In contrast to normal vessels, tumor vasculature is highly disorganized and the vessels do not fit into the normal classification of arterioles, capillaries or venules. They have irregular shapes, widely variable diameters and abnormal branching patterns (Less et al., 1991; Hashizume et al., 2000). The vessel wall may not always be lined by a homogenous layer of endothelial cells, but instead by cancer cells or a mosaic of cancer and endothelial cells (Hammersen et al., 1985; Chang et al., 2000). Further, the endothelial cells might form two, three or even several incomplete layers in which cell projections can extend between the layers and bridge the lumen (Hashizume et al., 2000).

It has previously been suggested that tumor vessels lack functional perivascular cells, and have a discontinuous or absent basement membrane (Benjamin et al., 1999). Recent work, however, indicates that endothelial cells, pericytes or basement membranes are present but abnormal (Morikawa et al., 2002). Pericytes on tumor vessels are clearly abnormal in shape, express atypical markers and have loose association with endothelial cells. Similar to endothelial cells, some pericytes overlap
Introduction

one another and form a sleeve around endothelial sprouts that is longer than the sprouts themselves (Morikawa et al., 2002).

The tumor vessel walls have numerous openings by endothelial fenestrae, vesicles and transcellular holes, as well as widened interendothelial junctions. These defects make tumor vessels hyperpermeable and leaky to circulating macromolecules (Gerlowski and Jain, 1986; Dvorak et al., 1988; Hobbs et al., 1998; Feng et al., 2000). Other abnormalities seen in tumor vessels include unusual avid binding properties, uptake of cationic liposomes, expression of integrins, different types of collagens, metalloproteinases, prostate-specific membrane antigens, and growth factors and receptors that differ from those present in normal vessels (Liu et al., 1997; Jussila et al., 1998; Lymboussaki et al., 1998; Thurston et al., 1998; Chang et al., 1999; Iurlaro et al., 1999; St Croix et al., 2000; Ran et al., 2002).

Tumors can acquire a blood supply by co-opting the existing vessels, through intussusception, sprouting angiogenesis and vasculogenesis. Tumor cells can initially grow around existing host vessels. When the tumor has grown to a size where the blood supply is not sufficient enough to support the outer layer of tumor cells, the vessels can regress, resulting in an avascular and hypoxic tumor. This hypoxia can induce robust new angiogenesis (Holash et al., 1999). Intussusceptive microvascular growth, also called vessel splitting, refers to an events where endothelial cells from opposite sides of the capillary protrude into the lumen and fuse, creating pillars across the capillary (Patan et al., 1996). In addition, vasculogenesis has been reported to contribute to the build up of new blood vessels in growing tumors (Asahara et al., 1999; Bolontrade et al., 2002). It has been suggested that tumors may form vascular channels without the involvement of endothelial cells (so called vasculogenic mimicry) but the incident and explanation of this phenomenon remains controversial (Maniotis et al., 1999; McDonald et al., 2000).

Angiogenesis is not only important for tumor growth but it also provides an efficient route of exit for tumor cells to leave the primary site and enter the blood stream (Liotta et al., 1974; Zetter, 1998) Long-term suppression of angiogenesis might become a
Angiostatic mechanisms of endogenous angiogenesis inhibitors

therapeutic option for induction of long-term remission by maintaining micro-
metastases in a state of dormancy (Holmgren et al., 1995; O'Reilly et al., 1996).

In order for the tumors to develop in size and metastatic potential, they have to switch
to an angiogenic phenotype by disturbing the local balance between proangiogenic and
antiangiogenic factors (Folkman, 1992; Dameron et al., 1994). The first molecule
identified as a purified angiogenic factor was basic fibroblast factor (bFGF) (Shing et
al., 1984). While bFGF exhibits its biological effect on a variety of cells, vascular
endothelial growth factor (VEGF) appears to be the most specific endothelial growth
factor. Since the discovery of angiogenic stimulators, the angiogenesis research has

Figure 2. Blood supply to tumors. See text for details.
heavily focused on pro-angiogenic growth factors. It took almost ten years before the first angiogenesis inhibitors were found.

1.4 ENDOGENOUS INHIBITORS

The first endogenous angiogenesis inhibitor to be associated with negative tumor regulation was thrombospondin-1 (Good et al., 1990). Since then, several endogenous inhibitors have been identified, including angiostatin, endostatin, tumstatin, vasostatin, serpin antithrombin, arrestin, and restin (O'Reilly et al., 1994; O'Reilly et al., 1997; Pike et al., 1998; O'Reilly et al., 1999a; Ramchandran et al., 1999; Colorado et al., 2000; Kamphaus et al., 2000; Maeshima et al., 2000). Interestingly, several endogenous angiogenesis inhibitors are proteolytic fragments of larger precursor proteins, such as angiostatin originating from plasminogen and endostatin from collagen XVIII. Thus, proteolytic processing appears to play critical dual roles in control of angiogenesis as proteases are required for invasion and migration of cells, and as some pro-angiogenic factors can be released by proteases from the extra cellular matrix (ECM) (Andreasen et al., 2000; Bergers et al., 2000). Among all endogenous inhibitors, angiostatin and endostatin seem to be the most promising.

1.5 ANGIOSTATIN, K1-5, AND OTHER KRINGLE FRAGMENTS

Angiostatin was discovered nearly 10 years ago as an internal fragment of plasminogen in serum and urine of tumor bearing mice (O'Reilly et al., 1994). Originally, angiostatin was referred to contain four of the disulphide-linked loops of plasminogen (Pgn) known as the kringle domains. However, in the literature angiostatin-fragments may contain either the first three or four kringle domains (K1-3 or K1-4). To even further complicate the terminology, kringles 1-5 of plasminogen (K1-5) are occasionally called angiostatin or angiostatin_4,5 as well. K1-5 consists of kringles 1-4 and 86% of kringle 5. Furthermore, an A61 form of angiostatin, referring to kringles 1-5 and part of kringle 5, has been reported. In addition, angiostatin exists in several different proteolytic fragments (discussed further in the proteases section), which may explain different activities of angiostatin reported by diverse research groups. It is important that readers keep in mind that the term angiostatin may actually refer to several different angiostatin related-proteins.
In vitro, angiostatin is relatively specific for endothelial cells or endothelial cell progenitors and inhibits their proliferation, but not the proliferation of other cell types, including tumor cells (O’Reilly et al., 1994; Cao et al., 1996; Dong et al., 1997; Stathakis et al., 1997; Ito et al., 1999). In addition, angiostatin inhibits endothelial cell migration, invasion and tube-formation in three-dimensional in vitro angiogenesis systems (Gately et al., 1996; Gately et al., 1997; Claesson-Welsh et al., 1998; Ji et al., 1998b; Stack et al., 1999; Tarui et al., 2002). Exceptions to this specificity for endothelial cells are some smooth muscle cells and neutrophils. At higher concentrations, angiostatin is able to inhibit aortic smooth muscle cell proliferation and migration induced by hepatocyte growth factor (HGF), probably by preventing the structurally homologous angiogenic factor HGF from binding to its cell surface receptor c-met (Walter and Sane, 1999). Neutrophil migration is attenuated by angiostatin, which is not surprising since neutrophils express angiomotin, a receptor involved in endothelial migration (Troyanovsky et al., 2001; Benelli et al., 2002). Additionally, in regard to proliferation, migration and tube-formation, angiostatin reduces sprouting in the mouse aortic ring assay (Hajitou et al., 2002) and interferes
Angiostatin with vessel network formation in the embryonic body model (Eriksson et al., 2003). Furthermore, in an experimental *in vitro* model angiostatin negatively regulates endothelial dependent vasodilation (Koshida et al., 2003).

*In vivo*, angiostatin suppresses neovascularization in the chick chorioallantoic membrane (CAM) assay and in the mouse cornea assay (O'Reilly et al., 1994; Gately et al., 1996; Morikawa et al., 2000). It also inhibits experimental primary tumor growth as well as angiogenesis dependent growth of metastases in mice (O'Reilly et al., 1994; O'Reilly et al., 1996; Gately et al., 1997; Cao et al., 1998). Recently, angiostatin or angiostatin-related fragments have been reported to inhibit pathological retinal angiogenesis of premature infants (Drixler et al., 2001) or hypoxia induced retinal neovascularization (Zhang et al., 2001). In a rat chronic infusion model of peritoneal dialysis, angiostatin improved peritoneal membrane dysfunction (Margetts et al., 2002), and it repressed experimentally induced arthritis in mice (Kim et al., 2002). In addition, angiostatin inhibits angiogenesis in obesity (Rupnick et al., 2002).

### 1.5.1 Naturally occurring angiostatin

While angiostatin was originally described as occurring in tumor-bearing mice, more recent studies have demonstrated that angiostatin and angiostatin-related proteins are found in humans. Soff et al. demonstrated that K1-5 (called angiostatin<sub>4.5</sub> by the group) is naturally occurring in normal human plasma at a concentration of approximately 6-12 nM. Markedly elevated levels were observed in ascites from patients with ovarian cancer as well as nonmalignant etiologies of ascites (Soff et al., 1999). In addition, it was shown by Cao et al. that angiostatin levels were significantly increased in the urine of cancer patients (Cao et al., 2000). Even though angiostatin fragments are detected in the urine of cancer patients, their prognostic importance are unknown. Angiostatin-related fragments might also play a role in preventing neovascularization in the hypoxic closed eye environment (Sack et al., 1999). In these experiments, tears collected after overnight closure contained elevated levels of plasminogen and various angiostatin-related fragments. Furthermore, angiostatin fragments were detected in tear fluid after surgical implantation of polymethyl methacrylate intrasomal corneal rings for correction of myopia (Lembach et al., 2001), and in the vitreous after retinal photocoagulation of
patients with proliferative diabetic retinopathy (Spranger et al., 2000). However, the increased levels of angiostatin after photocoagulation were not sufficient enough to neutralize the effects of high levels of VEGF. Angiostatin was found to be elevated during impaired production of nitric oxide in coronary angiogenesis (Matsunaga et al., 2002), indicating the consistency of concept that a balance between growth factors and angiogenesis inhibitors also control coronary angiogenesis and collateralization. Significantly elevated levels of angiostatin were found in bronchoalveolar lavage fluids from patients at risk for, and with early acute respiratory distress syndrome (ARDS) (Lucas et al., 2002). This is characterized by a disruption of the alveolar-capillary barrier, due to both an epithelial and an endothelial dysfunction. The same observation was done when volunteers were treated with endotoxin, suggesting that angiostatin may contribute to the endothelial damage observed in ARDS patients, possibly via an increased permeability of alveolar capillary barrier allowing for an intra-alveolar processing of plasminogen. The role of angiostatin has been demonstrated in a provoked murine inflammation model, and angiostatin has been found as a naturally occurring protein in chorioallantoic membrane of chicken (Falcone et al., 1998; Hatziapostolou et al., 2003) Altogether, these findings suggest that angiostatin is not a tumor specific inhibitor, but rather one of the key negative regulators of angiogenesis.

1.5.2 Kringle structure

A kringle structure describes a type of Scandinavian cookie or Danish pastry folded into two rings. This phrase was initially used to describe a triple loop structure linked by three pairs of disulphide bond present in prothrombin (Magnusson et al., 1975). The primary amino acid sequence of each kringle domain is composed of about 80 amino acids. In addition to the six conserved cysteine residues in their predicted positions, amino acids bordering the third and fourth cysteines are also highly conserved (see figure 4). Many proteins contain anything from one to several kringle domains. These proteins can be growth factors, receptors, proteinases or coagulation factors (reviewed in Cao et al., 2002). It is believed that these kringle structures mediate protein-protein interactions, such as plasminogen to fibrin or laminin (Lerch et al., 1980; Vali and Patthy, 1984; Moser et al., 1993).
The different kringles of angiostatin have been demonstrated to exhibit differential effects on suppression of endothelial growth and migration (Cao et al., 1996; Ji et al., 1998b). For example, the kringle 1 (K1) is a potent inhibitor of endothelial cell proliferation, whereas kringle 4 (K4) display low inhibitory activity (Cao et al., 1996). However, K4 has been reported to be very potent in inhibiting endothelial cell migration (Ji et al., 1998b.). What distinguish kringle 4 from other kringles are two clusters of positively charged lysine residues, adjacent to cysteine 22 and cysteine 80. These lysines expose a positively charged area in the three dimensional structure, and have been hypothesized to contribute to the loss of endothelial inhibitory activity (Cao et al., 1996; Cao et al., 2002). Another theory might be that K4, compared to other kringles, has a beta-sheet of amino acids bordering cysteine 51, in the middle of the
**Table 1. Kringle-containing proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nr of kringles</th>
<th>Antiangiogenesis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin</td>
<td>2</td>
<td>Yes</td>
<td>(Magnusson et al., 1975)</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>5</td>
<td>Yes</td>
<td>(Sottrup-Jensen et al., 1975; Sottrup-Jensen et al., 1978)</td>
</tr>
<tr>
<td>uPa(^1)</td>
<td>1</td>
<td>N.D.</td>
<td>(Gunzler et al., 1982)</td>
</tr>
<tr>
<td>tPa(^2)</td>
<td>2</td>
<td>Yes</td>
<td>(Pennica et al., 1983)</td>
</tr>
<tr>
<td>Factor XII</td>
<td>1</td>
<td>N.D.</td>
<td>(Cool et al., 1985; McMullen and Fujikawa, 1985)</td>
</tr>
<tr>
<td>Apolipoprotein(a)</td>
<td>38</td>
<td>Yes</td>
<td>(Eaton et al., 1987; McLean et al., 1987)</td>
</tr>
<tr>
<td>HGF(^3)/SF(^4)</td>
<td>4</td>
<td>Yes</td>
<td>(Nakamura et al., 1989)</td>
</tr>
<tr>
<td>MSP(^5)/HGF like protein</td>
<td>4</td>
<td>N.D.</td>
<td>(Han et al., 1991)</td>
</tr>
<tr>
<td>HGF(^3) activator</td>
<td>1</td>
<td>N.D.</td>
<td>(Miyazawa et al., 1993)</td>
</tr>
<tr>
<td>Kremen</td>
<td>1</td>
<td>N.D.</td>
<td>(Nakamura et al., 2001)</td>
</tr>
<tr>
<td>Neurotrypsin/ Motopsin</td>
<td>1</td>
<td>N.D.</td>
<td>(Gschwend et al., 1997)</td>
</tr>
<tr>
<td>(Yamamura et al., 1997)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHBP(^6)</td>
<td>1</td>
<td>N.D.</td>
<td>(Choi-Miura et al., 1996)</td>
</tr>
<tr>
<td>Serine protease (<strong>Hermandid momus</strong>)</td>
<td>1</td>
<td>N.D.</td>
<td>(Arnold et al., 1997)</td>
</tr>
<tr>
<td>ROR 1&amp;2</td>
<td>1</td>
<td>N.D.</td>
<td>(Masiakowski and Carroll, 1992)</td>
</tr>
<tr>
<td><strong>Drosophila</strong> neurospecific receptor kinase</td>
<td>1</td>
<td>N.D.</td>
<td>(Oishi et al., 1997)</td>
</tr>
<tr>
<td><strong>Drosophila</strong> receptor tyrosine kinase</td>
<td>1</td>
<td>N.D.</td>
<td>(Wilson et al., 1993)</td>
</tr>
<tr>
<td><strong>C.elegans</strong> ROR receptor Tyrosine kinase</td>
<td>1</td>
<td>N.D.</td>
<td>(Forrester et al., 1999)</td>
</tr>
<tr>
<td><strong>Musk</strong>(^7) (<strong>Torpedo, Xenopus</strong>)</td>
<td>1</td>
<td>N.D.</td>
<td>(Jennings et al., 1993)</td>
</tr>
<tr>
<td>(Fu et al., 1999a)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Urokinase-type plasminogen activator, \(^2\)Tissue-type plasminogen activator
\(^3\)Hepatocyte growth factor \(^4\)Scatter factor \(^5\)Macrophage-stimulating protein \(^6\)Plasma hyaluronan- binding protein \(^7\)Muscle specific tyrosine kinase  
N.D. = Not Determined
conserved amino acids (Patthy et al., 1984). Since these amino acids are highly conserved in all kringle structures, they seem to be important for their function. The altered structure in K4 may have resulted in loss of its function. Kringle 5 (K5) of plasminogen seems to be the most potent of all kringles in inhibiting proliferation (Cao et al., 1997). Furthermore, K5 seems to be a potent inhibitor of migration, and an inducer of apoptosis (Ji et al., 1998a; Lu et al., 1999).

Not only kringles of plasminogen have been shown to inhibit angiogenesis. In addition, kringle-domains in apolipoprotein a (apo(a)), hepatocyte growth factor (HGF), urokinase plasminogen activator (uPA) and kringle-2 of pro-thrombin show angiostatic properties (Lee et al., 1998; Rhim et al., 1998; Trieu and Uckun, 1999; Kuba et al., 2000a; Kuba et al., 2000b; Xin et al., 2000; Schulte et al., 2001; Kim et al., 2003). The question remains if all kringles have anti-angiogenic properties.

1.5.3 Proteases

Several different proteolytic processes can cleave plasminogen/plasmin at various sites, resulting in a range of kringle-containing fragments collectively termed angiostatin. The differences in angiostatin structures created by different proteases, may explain, in part, the differences in experimental results from different research groups.

After the discovery of angiostatin, the immediate question raised was how angiostatin is generated. The earliest discovery of mechanism was plasmin autodigestion. This process is dependent on the activity of urokinase plasminogen activator (u-PA) and free sulfhydryl donor or plasmin reductase (Gately et al., 1997; Stathakis et al., 1997). Recent reports have identified that phosphoglycerate kinase and annexin II can function as a plasmin reductase (Lay et al., 2000; Kwon et al., 2002). Other groups have reported that various metalloproteinases can convert plasminogen to angiostatin, including gelatinase A (MMP-2), stromelysin (MMP-3), matrilysin (MMP-7) gelatinase B/ Type IV Collagenase (MMP-9) and macrophage-derived metallo-elastase (MMP-12 or MME) (Dong et al., 1997; Patterson and Sang, 1997; Lijnen et al., 1998; O'Reilly et al., 1999b). Additional proteases that have been reported to create angiostatin-related fragments are the proteinase prostate-specific antigen (PSA), Cathepsin D, 13 kDa serine protease and 24 kDa endopeptidase.
**Angiostatic mechanisms of endogenous angiogenesis inhibitors**

**Tabel 2. Proteases that cleaves angiotatin from plasminogen**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Product</th>
<th>Cleavage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2/ Gelatinase A</td>
<td>K1-3?</td>
<td>Tyr80-?</td>
<td>(O'Reilly et al., 1999b)</td>
</tr>
<tr>
<td>MMP-3/ Stomelysin</td>
<td>K1-4</td>
<td>Asn 60-Pro447</td>
<td>(Lijnen et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>K5</td>
<td>Val448-Pro544</td>
<td></td>
</tr>
<tr>
<td>MMP-7/ Matrilysin</td>
<td>K1-4</td>
<td>Lys78-Pro446</td>
<td>(Patterson and Sang, 1997)</td>
</tr>
<tr>
<td>MMP-9/ GelatinaseB/ Type IV Collagenase</td>
<td>K1-4</td>
<td>Lys78-Pro447</td>
<td>(Patterson and Sang, 1997)</td>
</tr>
<tr>
<td>MMP-12/MME</td>
<td>K1-?</td>
<td>N.I.</td>
<td>(Dong et al., 1997)</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>K1-4</td>
<td>Phe75-Leu451</td>
<td>(Morikawa et al., 2000)</td>
</tr>
<tr>
<td>PSA</td>
<td>K1-4</td>
<td>Val78-Glu439</td>
<td>(Heidtmann et al., 1999)</td>
</tr>
<tr>
<td>Elastase</td>
<td>K1-3</td>
<td>Tyr80-Val338/354</td>
<td>(Sottrup-Jensen et al., 1978)</td>
</tr>
<tr>
<td>Elastase</td>
<td>K4</td>
<td>Val 355-Ala440</td>
<td>(Sottrup-Jensen et al., 1978)</td>
</tr>
<tr>
<td></td>
<td>K1-4</td>
<td>Tyr80-Ala440</td>
<td>(Takada et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>K5</td>
<td>Val449/Pro452-Ala544</td>
<td>(Cao et al., 1997)</td>
</tr>
<tr>
<td>Elastase + pepsin</td>
<td>K5</td>
<td>Val442-Phe545</td>
<td>(Novokhatny et al., 1984)</td>
</tr>
<tr>
<td></td>
<td>K1</td>
<td>N.I.</td>
<td>(Matsuka et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>K2-K3</td>
<td>N.I.</td>
<td></td>
</tr>
<tr>
<td>Elastase + chymotrypsin</td>
<td>K1</td>
<td>Tyr80-Glu166</td>
<td>(Lerch et al., 1980)</td>
</tr>
<tr>
<td>Plasmin autodigestion</td>
<td>p22</td>
<td>Lys78-Lys180</td>
<td>(Kwon et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>K1-4?</td>
<td>Lys 77.?-</td>
<td>(Falcone et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>A61</td>
<td>Lys78-Lys468</td>
<td>(Kassam et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>K1-4, K1-5</td>
<td>Lys78-?</td>
<td>(Stathakis et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>K1-5</td>
<td>Lys78-arg530</td>
<td>(Cao et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>K1-5</td>
<td>Lys 78.-?</td>
<td>(Gately et al., 1997)</td>
</tr>
<tr>
<td>13KD serine protease</td>
<td>K1-5?</td>
<td>Lys78.-?</td>
<td>(Li et al., 2000)</td>
</tr>
<tr>
<td>Elastase + <em>Staphylococcus Aureus</em> V8 protease</td>
<td>K1</td>
<td>Tyr80-Glu167</td>
<td>(Motta et al., 1986)</td>
</tr>
<tr>
<td>Elastase + streptokinase reduction+alkylation</td>
<td>K5</td>
<td>Val442-Arg560</td>
<td>(Vali and Patthy, 1984)</td>
</tr>
<tr>
<td>24KD endopeptidase</td>
<td>K1-4</td>
<td>Val79-Ser441</td>
<td>(Lijnen et al., 2000)</td>
</tr>
</tbody>
</table>
Angiostatin
(Heidtmann et al., 1999; Li et al., 2000; Lijnen et al., 2000; Morikawa et al., 2000).
Different angiostatin-fragments created by various proteases are shown in table 2.

Although plasmin and metalloproteinases appear to regulate angiogenesis inhibitors,
they are also involved in positive regulation of angiogenesis. For example, plasmin is
considered to be a key protease in tumor invasion of neighboring tissues (Stack et al.,
1999; Andreasen et al., 2000). Binding of MMP-2 to integrin αvβ3 on the tip of
sprouting vessels appears to facilitate extracellular matrix degradation and subsequent
new blood vessel formation (Brooks et al., 1996). Membrane type-1 matrix
metalloproteinase (MT-MMP1) can degrade cross-linked fibrin gels and directly
mediate invasive events and is shown to be essential protease for neovascularization
(Hiraoka et al., 1998; Zhou et al., 2000). Furthermore, metalloproteinases-9 is known to
release immobilized angiogenic factors from the matrix (Bergers et al., 2000)

1.5.4 Angiostatin binding proteins / receptors

The two only known “receptors” for angiostatin are F₀F₁-ATP synthase and angiomotin
(Moser et al., 1999; Moser et al., 2001; Troyanovsky et al., 2001). A fascinating study
by Moser et al. demonstrated that angiostatin, but not plasminogen, binds to an ATP
synthase on the surface of endothelial cells, with the catalytic subunit located
extracellularly (Moser et al., 1999). This is a controversial finding since ATP synthase is
normally located on the mitochondrial membrane. Inhibition of this enzyme by a
specific antibody impairs the antiproliferative effect of angiostatin (Moser et al., 1999).
Finally, angiostatin is able to inhibit ATP synthase activity on endothelial cells (Moser
et al., 2001).

Angiomotin is a cell surface protein, but does not appear to be a signal transducing
receptor. However, the cells that contain angiomotin can bind and internalize
angiostatin. This results in focal adhesion kinase (FAK) activation and inhibition of
migration and tube-formation. It is possible that there are accessory protein(s) that bind
to angiomotin and mediate the downstream signals. This interaction might occur
through PDZ-binding motif since deletion of this domain completely attenuates the pro-
migratory activity of angiomotin (Bratt et al., 2002; Levchenko et al., 2003).
In addition to ATP synthase and angiomotin, angiostatin can specifically interact with \( \alpha \beta 3 \) integrins, which have been shown to be critical for angiogenesis (Brooks et al., 1998; Tarui et al., 2001). However, it is not totally clear how this interaction modulates angiogenesis. Free angiostatin also binds to and sequesters NG2-proteoglycan expressed by pericytes. This might promote neovascularization by neutralizing the inhibitory activity of angiostatin on endothelial cell proliferation and migration (Goretzki et al., 2000). Other binding proteins for angiostatin are, for example, tissue-type plasminogen activator (tPA) and CD26, both of which are reported to be involved in cellular invasion of tumor cells (Stack et al., 1999; Gonzalez-Gronow and Pizzo, 2001). Finally, angiostatin has been reported to bind annexin II, although the importance of this finding remains to be seen (Tuszynski et al., 2002).

### 1.5.5 Mechanism

Although angiostatin was discovered several years ago, little knowledge has been gained in regard to its molecular mechanism. Some of the mechanisms reported are occasionally controversial, possibly due to variations in endothelial cell types and properties and/or differences in experimental design. For example, one report demonstrates that an adenovirus-produced angiostatin (K1-3) selectively prevents the \( G_2/M \) phase transition in endothelial cells (Griscelli et al., 1998), which may explain the effect of angiostatin on proliferation. However, several other groups have not found recombinant angiostatin to affect endothelial cell cycle checkpoints, neither \( G_0/G_1 \), nor \( G_2/M \) (Claesson-Welsh et al., 1998; Lucas et al., 1998; Luo et al., 1998). Rather than affecting cell cycle, it has been found by independent groups that angiostatin is able to induce endothelial cell apoptosis both in vitro endothelial cell culture systems and in vivo, and this induction seems to be caspase-3 dependent (Claesson-Welsh et al., 1998; Lucas et al., 1998; Lu et al., 1999; Volpert et al., 2002). Microchip technology has revealed that this apoptosis induction might also involve downregulation of c-fos, MAPK-2 and Bcl-2, and upregulation of mxil, bad, bax, and p53 by angiostatin treatment (Shichiri and Hirata, 2001). Furthermore, it is possible that angiostatin mediates apoptosis by transient increase in ceramide, which results in activation of RhoA, ROCK and LIMK-2, proteins involved in actin stress fiber reorganization, further leading to detachment and death (Gupta et al., 2001). Although multiple forms of angiostatin have been found to induce endothelial cell apoptosis at various
concentrations, the apoptotic signaling pathways triggered by angiostatin and the role of angiostatin-induced apoptosis in mediating in vivo angiostatic action of angiostatin are still not clear.

Although angiostatin is an endothelial cell specific inhibitor, it appears to be able to down-regulate VEGF secretion from tumor cells. However, this inhibition seems to be tumor type specific (Sun et al., 2001; Gao et al., 2002; Hajitou et al., 2002). Even K5 alone can downregulate VEGF secretion from endothelial cells, and this inhibition is pronounced in hypoxic conditions together with upregulation of pigment epithelium derived factor (PEDF) (Gao et al., 2002). However, neither angiostatin, nor K5 appear to be capable of interfering with VEGF binding to its receptor (Claesson-Welsh et al., 1998; Gao et al., 2002). One report has implied that angiostatin can only counteracts HGF-induced proliferation, migration and MAPK phosphorylation (Wajih and Sane, 2002). This contradicts earlier studies which reported that angiostatin counteract angiogenic properties of FGF and VEGF (O’Reilly et al., 1994; Cao et al., 1996; Gately et al., 1996; Dong et al., 1997; Statakhis et al., 1997; Claesson-Welsh et al., 1998; Redlitz et al., 1999). Other signal transduction studies have indicated that angiostatin reduces bFGF- and VEGF- induced phosphorylation of ERK-1 and ERK-2 in human dermal microvascular cells (HMDEC). However, this effect is not seen by others, neither in HMDEC nor in bovine capillary endothelial cells (BCE) (Claesson-Welsh et al., 1998; Redlitz et al., 1999; Eriksson et al., 2003). Instead, angiostatin is capable of attenuating VEGF-induced activation of focal adhesion kinase (FAK) (Claesson-Welsh et al., 1998).

Angiostatin regulates endothelial dependent vasodilation by decreasing VEGF-induced hsp90 interactions with endothelial nitric oxide synthase (eNOS) (Koshida et al., 2003). This might be coincidental with the fact that angiostatin is able to attenuate VEGF-mediated down-regulation of caveolin-1 (Liu et al., 1999), since caveolin-1 is a negative regulator of eNOS, whereas hsp90 is a positive regulator. Hsp90 can recruit Akt, which phosphorylates eNOS and thereby increases its activity. eNOS itself is a positive regulator of angiogenesis by producing nitric oxide (NO), which is an important signal transducer of endothelial cell migration and survival (Kim et al., 1997; Murohara et al., 1999; Mannick et al., 2001).
Finally, angiostatin upregulates E-selectin and increases intracellular Ca\textsuperscript{2+}-concentrations in endothelial cells (Luo et al., 1998; Jiang et al., 2001). Moreover, at tumor-like low pH angiostatin is able to induce intracellular acidosis and anoikis and is more effective in displaying its angiostatic properties than at normal pH (Wahl and Grant, 2000; Wahl et al., 2002). In addition to differences in cell types and angiostatin fragments used, it is possible that even slight differences in pH obtained in different laboratories contributes to variations in experimental results. Nevertheless, angiostatin has been found to be a potent angiogenesis inhibitor, both \textit{in vitro} and \textit{in vivo}, as well as exhibiting multiple effects on endothelial cells.

1.6 ADIPONECTIN

Adiponectin is a \sim30kDa protein secreted from differentiated adipocytes. Adiponectin is also referred to as Acrp30 (adipocyte complement-related protein of 30kDa), AdipoQ, apM1 (adipose most abundant gene transcript 1), and GBP28 (gelatin-binding protein 28) (Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996). All of these names are currently used in the literature.

Human adiponectin is a polypeptide of 244 amino acids with a secretory signal sequence, a small non-helical region, a collagen-like fibrous domain, and a complementary C1q-like globular domain. Adiponectin do not usually exist as monomers, but rather form trimers, hexamers and oligomers through interactions between the collagenous domains. Although there is no detailed information of about the structure of adiponectin available at this time, the crystal structure of its globular domain shows an unexpected homology with tumor necrosis factor (TNF)-\alpha (Shapiro and Scherer, 1998).

Adiponectin is exclusively expressed by adipocytes and is abundantly present in the circulation. Concentrations found in the plasma from healthy humans, range from 2 - 17 \textmu g/ml and this corresponds approximately to 0.01\% of total plasma protein (Arita et al., 1999; Okamoto et al., 2000). Female mice display higher levels and more complex oligomers in serum than male mice (Arita et al., 1999; Pajvani et al., 2003).
Furthermore, the expression of adiponectin is negatively correlated with fat mass and decreases in obese persons and patients with coronary artery disease or type II diabetes (Arita et al., 1999; Ouchi et al., 1999; Hotta et al., 2000; Hotta et al., 2001).

### 1.6.1 Function

Adiponectin is an important regulator of insulin sensitivity, which regulates energy homeostasis. Acute treatment of mice with adiponectin significantly decreases the elevated levels of plasma free fatty acids and glucose caused by a high-fat/high-sucrose meal. This effect is probably due to an increase in fatty acid oxidation in the muscle tissue, as well as an increased insulin sensitivity (Fruebis et al., 2001; Yamauchi et al., 2003). Further, one report suggested that treatment of mice on high-fat diets with adiponectin, results in profound and sustainable weight reduction without affecting the amount of food consumed (Fruebis et al., 2001). However, adiponectin-overexpressing mice lose weight only if food intake is restricted. This indicates that adiponectin increases the energy expenditure, and this energy expenditure is compensated by increased food intake (Yamauchi et al., 2003).

It is clear that adiponectin is important in pathological conditions, but the adiponectin system may not be required under physiological conditions. Deletion or overexpression of adiponectin in mice does not result in developmental abnormalities, or any changes in the body weight and organ histology (Maeda et al., 2002; Yamauchi et al., 2003). Adiponectin null-mice display normal insulin sensitivity under a regular diet, however, when placed on a high fat/high sucrose diet, the mice exhibit signs of severe insulin resistance. In addition, adiponectin-deficient mice display severe neointimal thickening and increased proliferation of vascular smooth muscle cells in mechanically injured arteries. This proliferation is attenuated by adenovirus-mediated addition of adiponectin (Matsuda et al., 2002). Furthermore, overexpression of the globular domain of adiponectin in ApoE-deficient mice protected the mice from atherosclerosis (Yamauchi et al., 2003). This effect could be due to several effects of adiponectin. First, adiponectin generally attenuates monocyte attachment to endothelial cells by reducing the expression of the adhesion molecules ICAM-1 (intracellular adhesion molecule-1), VCAM-1 (vascular adhesion molecule-1) and E-selectin on endothelial cells (Ouchi et
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(Alitalo, 1999; Ouchi et al., 2000). Secondly, adiponectin suppresses lipid accumulation in monocyte-derived macrophages through the suppression of macrophage scavenger receptor expression (Ouchi et al., 2001; Yamauchi et al., 2003). Thus, adiponectin inhibits macrophage-to-foam cell transformation, a step in atherosclerosis pathogenesis (Ouchi et al., 2001). Thirdly, adiponectin suppresses both the expression and pro-inflammatory action of TNF-\(\alpha\) (Yokota et al., 2000; Maeda et al., 2002). Finally, adiponectin inhibits proliferation of vascular smooth muscle cells and myelomonocytic progenitor cells. In cultured smooth muscle cells (SMC) adiponectin inhibits proliferation induced by platelet-derived growth factor (PDGF)-AA, PDGF-BB, epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), and bFGF (Arita et al., 2002; Matsuda et al., 2002). Further, adiponectin inhibits PDGF-BB- and HB-EGF-induced migration in SMCs and HB-EGF secretion from endothelial cells (Arita et al., 2002; Matsuda et al., 2002). Adiponectin can directly bind to PDGF-BB and thereby inhibits its binding to SMCs (Arita et al., 2002). In addition, adiponectin inhibits proliferation of myelomonocytic progenitor cells and induces apoptosis in these cells. This apoptosis induction is connected with downregulation of anti-apoptotic bcl-2 and bcl-xl, but does not involve pro-apoptotic bak, bax or p53 (Yokota et al., 2000).

When an artery is injured by balloon angioplasty, adiponectin penetrates quickly into the subendothelial space of the vascular wall where adiponectin binds specifically to collagen I, II and V and may play a role as a scaffold for newly formed collagen (Nakano et al., 1996; Okamoto et al., 2000). The same might be true for myocardial remodeling after ischaemic injury (Ishikawa et al., 2003). Thus, adiponectin appears to be an important protein that regulates energy homeostasis, the immunological responses and vascular remodeling under pathological conditions.
2 SENESCENCE

2.1 REPLICATIVE SENESCENCE

After a critical number of population doublings (PDLs), cells stop dividing and enter a state known as replicative senescence. Replicative senescence was first described 40 years ago in human fibroblasts (Hayflick and Moorehead, 1961). Since then, many cell types have been shown to have finite life spans even with ideal growth conditions. The number of divisions that normal cells complete before they senesce depends on the species, age, genetic background of the donor, and growth conditions, as well as the particular cell type. Replicative senescence seems to be particularly stringent in human cells which, unlike many rodent cells, rarely become spontaneously immortalized. The only known exceptions to finite replication are the germ-line cells, certain stem cells, and tumor cells, which all appear to be able to replicate continuously.

![Diagram](image_url)

*Figure 5. Telomeres and the end-replication problem. Telomeres protect chromosomes from endonuclease degradations and fusions, and position chromosomes during mitosis. Since replication is initiated by primers in the 5' direction, a short piece of the telomeres are left unreplicated. This leads to telomere shortening during each division and is considered as a "mitotic clock". Modified figure from Blackburn, Scientific American, February 1996.*

2.2 SENESCENT PHENOTYPE

Senescent cells differ in a number of ways from non-senescent cells (reviewed in Stanulis-Praeger, 1987; Cristofalo and Pignolo, 1993; Campisi, 2000). Morpho-
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logically senescent cells are larger and more flattened with increased ratio of cytoplasm/nucleus (see paper IV in thesis). Senescent cells grow more slowly and are finally growth arrested irreversibly in the G1 phase of the cell cycle. This growth arrest can not be overridden by physiological mitogens, due to a change in expression of growth inhibitory proteins (Kaftory et al., 1978; Stein et al., 1990; Shay et al., 1991). Senescent cells often express a marker for senescence, senescence associated β-galactosidase (SA-β-gal), and they have an increased lysosomal biogenesis as well as decreased rates of protein synthesis and degradation (Macieira-Coelho and Lima, 1973; Kaftory et al., 1978; Stanulis-Praeger, 1987; Dimri et al., 1995). Furthermore, cell adhesion and plating efficiency can be decreased in senescent cell cultures (Azencott and Courtois, 1974; Couzin, 1978; West et al., 1989). Another feature that distinguishes senescent from presenescent cells is their resistance to programmed cell death (Wang, 1995). The mechanism by which senescent cells resist apoptotic death is not well understood, but this resistance seems to involve a failure to suppress bcl-2, and c-Jun NH2-kinases, ERKs and p38 MAPKs (Afshari et al., 1993; Wang, 1995; Suh, 2001; Suh and Park, 2001).

Changes in gene expression in senescent cells can be cell-type specific. For example, senescent human endothelial cells overexpress interleukin-1α and the cell adhesion molecule ICAM-1 (Maier et al., 1990; Maier et al., 1993). Bovine endothelial cells have been shown to downregulate the enzyme activity of angiotensin-converting (Del Vecchio and Smith, 1982). Moreover, senescent fibroblasts have increased expression of proteases, such as collagenase and stromelysin, and reduced expression of tissue inhibitor of metalloproteinase 1 and 3 (West et al., 1989; Millis et al., 1992; Wick et al., 1994).

2.3 INDUCERS OF SENESCENCE

The most important inducer of senescence is believed to be telomere shortening. Telomeres are the ends of chromosomes consisting of a repetitive DNA sequence and specialized proteins. In humans and other vertebrates, this sequence consists of repeats of TTAGGG. Telomeres are essential for chromosome stability and function as they protect chromosomes from degradation and fusion, contribute to the architecture of the nucleus and, in some organisms, function in gene expression by telomere silencing.
Senescence (Bacchetti, 1996). During DNA replication, the DNA polymerase moves uni-directionally and requires a primer for initiation. This leaves short pieces of DNA unreplicated in each round of DNA replication, that are further shortened by exonucleases, reducing their length by a total of 50-200 base pairs (Olovnikov, 1973). After a number of cell doublings, the telomere length has reached a point where the cells can no longer divide and, possibly as a result thereof, the senescent machinery is activated. This state is called replicative senescence. In support of this theory, cells in culture shorten their telomeres as they approach senescence. In addition, cells isolated from differently aged organisms might have, but not always, direct correlations between DNA length and replicative life span in culture (Harley et al., 1990; Hastie et al., 1990; Allsopp et al., 1992). Furthermore, introduction of human telomerase reverse transcriptase (hTERT) alone into primary human skin fibroblasts, retinal pigment epithelial cells and endothelial cells has been found to result in immortalization of these cells (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Yang et al., 1999).

How continual loss of telomeres activates the senescent machinery is not known. It is possible that short telomeres themselves, or end-to-end-fusions of chromosomes due to short telomeres, signal for DNA damage (Chin et al., 1999; Blackburn, 2000). In addition, due to short telomeres, proteins that protect the chromosomal ends from DNA degradation and end-to-end-fusions might lose their binding ability. For example, elimination of the telomere end-capping protein Ku86, involved in DNA double-strand break repair, leads to end-to-end-fusions and a premature, senescent-like growth arrest (Espejel and Blasco, 2002). Alternatively, telomeres could bind or sequester transcription factors, that activate or repress a range of genes (Campisi, 1997). One final explanation is that telomere position effect could silence gene expression reversibly by a mechanism which depends on both telomere length and the distance to genes (Baur et al., 2001).

Increasing amount of evidence suggests that DNA damage can induce a phenotype very similar to the phenotype seen in replicative senescence (Bladier et al., 1997; Chen et al., 1998; Robles and Adami, 1998; te Poele et al., 2002). Such damage, for instance, can be caused by radiation or hydrogen peroxide. In addition, inappropriate oncogene expression or mitogenic signals can induce a senescent-like phenotype. For example,
both Ras and Raf induce phenotypes similar to replicative senescence, including flattened morphology, SA-β-gal activity and induction of p16 INK4a (p16) (Serrano et al., 1997; Zhu et al., 1998).

2.4 MEDIATORS OF SENESCENCE

2.4.1 The p16INK4a/Rb pathway

A number of cell cycle regulatory proteins have been proposed to transduce senescence-inducing signals or mediate cell entrance into senescence. The most well-known and intensively studied is probably p16, which is progressively upregulated during the accumulation of population doublings (Alcorta et al., 1996; Palmero et al., 1997; Watanabe et al., 1997; Zindy et al., 1997; Huschtscha and Reddel, 1999; Munro et al., 1999). Consistent with the role of p16 in senescence, inactivation of the p16/Rb pathway results in lifespan extension and expression of p16 in immortalized human fibroblasts induces a senescent-like growth arrest (Loughran et al., 1996; Brenner et al., 1998; Vogt et al., 1998). Similarly, viral oncoproteins such as the SV40 large T antigen, the adenovirus E1a protein, or the herpes virus E7 protein, inactivate the growth suppressive functions of Rb (Retinoblastoma protein) and thereby facilitate immortalization (DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1989).

The role of p16 is to inhibit the cyclin dependent kinases (CDK) 4-6 and thereby participate in maintaining Rb in its unphosphorylated, growth-suppressive state. In its unphosphorylated, or hypophosphorylated, forms Rb associates with several transcription factors, such as the E2F proteins, silencing their transactivation functions. Upon phosphorylation, Rb is released, allowing E2F factors to turn on the expression of crucial cell cycle proteins such as cyclins E and A, followed by DNA synthesis (reviewed by Sherr, 1996), see figure 6.

As a tumor suppressor, p16 plays an important role, and it is inactivated in a large proportion of human tumors by deletion, mutation or hypermethylation (Caldas et al., 1994; Kamb et al., 1994; Okamoto et al., 1994; Cairns et al., 1995; Merlo et al., 1995). In addition to p16, the CDK4 gene or cyclin D are often amplified or mutated, so
making them resistant to p16-mediated inhibition (Jiang et al., 1992; Piccinin et al., 1997; Gorbunova et al., 2002). Furthermore, loss of Rb expression or protein function has been found in tumors, most commonly known in retinoblastoma (Lee et al., 1987). Over all, the p16INK4a/Rb pathway plays a crucial role as a major tumor suppressor pathway.

2.4.2 The p19ARF/p53/p21Cip1 pathway

A second gene implicated in senescence is p19ARF (p19) that is encoded by a partially overlapping alternative reading frame at the INK4a locus (Kamijo et al., 1997; Jacobs et al., 2000). When referring specifically to the human version, p19 is also called p14ARF. The p19 gene is upregulated during senescence in murine fibroblasts (Kamijo et al., 1997; Zindy et al., 1998) but not in human keratinocytes (Munro et al., 1999). This could indicate that the pathway mediating senescence is species- and/or cell-type specific. Deletion of p19 in mouse embryo fibroblasts (MEF) or repression of p19 expression by TBX2, a transcription factor and a repressor, has been shown to delay senescence (Kamijo et al., 1997; Jacobs et al., 1999; Jacobs et al., 2000). Furthermore overexpression of Bmi-1 in primary MEFs results in downregulation of both p16 and p19, causing an extended life span (Jacobs et al., 1999).

To regulate the cell cycle machinery, p19 binds directly to and sequesters MDM2, inhibiting the capacity of MDM2 to induce degradation of the p53 tumor suppressor protein. The p53 protein is a transcription factor that mediates cell-cycle arrest via upregulation of the CDK inhibitor p21Cip (p21). p21 inhibits the phosphorylation of Rb by cyclin D/CDK4 and cyclin E/CDK2 to block cell cycle progression. Alternatively it can act directly on PCNA to arrest the cell proliferation (for details, see figure 6).
Rather than increased expression as in the case of p16, the transcriptional activity of p53 is progressively elevated with increasing numbers of cell doublings (Afshari et al., 1993; Atadja et al., 1995; Bond et al., 1996). Induced expression of p53 in some cancer cell lines results in irreversible cell growth arrest with characteristics of replicative senescence (Wang et al., 1998b). Further, treatment of cells with a combination of p53 and Rb antisense oligonucleotides delays senescence, as well as expression of onco-
proteins such as SV40 large T antigen or herpes virus E6 (DeCaprio et al., 1988; Hara et al., 1991). In a number of cellular systems, the protein levels of p21 are elevated when the cells approach senescence, but decrease when the cells enter the senescent state (Tahara et al., 1995; Alcorta et al., 1996; Stein et al., 1999). In addition, the p21 gene was found to be a senescence-inducing gene in an expression screening (Noda et al., 1994). Whether or not p21 is essential for the establishment of senescence has to be further examined. Thus, disruption of the p21 gene allows cells to avoid senescence, although cells that lack p21 still undergo senescence (Brown et al., 1997; Pantoja and Serrano, 1999).

The role of p19 as a tumor suppressor is not clear. Since both p16 and p19 share the same locus, deletions and mutations can affect both p16 and p19. However, there are mutations that specifically target p16, but not p19 (Ruas and Peters, 1998). The role of the tumor suppressor protein p53 is much more evident since it is inactivated in approximately half of all human cancers. MDM2 is frequently amplified or overexpressed in sarcomas and in some other human cancers (reviewed by Momand et al., 1998). Concurring with the role of MDM2 as a repressor of p53 function, tumors overexpressing MDM2 do not have detectable p53 levels and do not carry mutations in the p53 gene. In contrast, alteration of p21 is rarely detected in cancer, and p21 knockout mice show no increased risk for tumor formation (Brugarolas et al., 1995; Deng et al., 1995; Terry et al., 1996).
3 TELOMERASE

The preservation of telomeric repeats during several cell division cycles requires telomerase, a telomere-specific ribonucleoprotein reverse transcriptase. The telomerase is a complex, consisting of an RNA component, telomerase reverse transcriptase (TERT) and telomere-associated proteins such as TAP-1 (Nugent and Lundblad, 1998; Blackburn, 2001). Telomerase is active during human embryogenesis, but in adults the expression was previously thought to be limited to germ-line cells and some stem cells. Now it is becoming more apparent that telomerase activity is present in many normal cells, including endothelial cells during early population doublings, vascular smooth muscle cells, hair follicles, endometrium during late-phase of proliferation, lymphocytes, and cells in the regenerative basal layer of the epidermis in human skin (Broccoli et al., 1995; Harle-Bachor and Boukamp, 1996; Norrback et al., 1996; Taylor et al., 1996; Hsiao et al., 1997; Kyo et al., 1997; Ramirez et al., 1997; Tanaka et al., 1998; Minamino et al., 2001). It is possible that more sensitive assays would detect weak telomerase activity even in other organs and tissues.

Telomerase lengthens the telomeres by copying a template sequence carried with its internal RNA. Single stranded telomeric TTAGGG repeats are added to the 3’ ends of the DNA. Telomerase is a central player in the maintenance of steady state telomere length and is, therefore, a major target for both positive and negative regulators.

3.1 REGULATION OF TELOMERASE

Several studies propose that TERT is the limiting factor of telomerase activity since this component is downregulated in normal somatic cells (Weinrich et al., 1997; Bodnar et al., 1998; Vaziri and Benchimol, 1998). The oncogene Myc is known to be an important activator of telomerase (Wang et al., 1998a) and there are two important Myc binding sites, referred to as E boxes, present in the hTERT core promoter (Wu et al., 1999; Gunes et al., 2000; Kyo et al., 2000). Moreover, c-Myc induction of hTERT expression is fast and independent of cell proliferation or additional protein synthesis, consistent with direct transcriptional activation by c-Myc. In addition to c-Myc, sp1 has
been reported to cooperate with c-Myc to activate hTERT transcription (Kyo et al., 2000). Another factor which is believed to upregulate hTERT is Bcl-2, since overexpression of Bcl-2 increases telomerase activity (Mandal and Kumar, 1997; Johnson et al., 1999). Finally, the TERT gene can be induced by NF-κB (Yin et al., 2000).

The Rb family of proteins appears to control the telomere length, since knockout MEFs increased their telomere length during each cell division (Xu et al., 1997; Garcia-Cao et al., 2002). Growth factors and the growth substrate may provide signals that regulate telomerase activity and/or hTERT expression. For example, bFGF causes an increase in telomerase activity and proliferation of mouse neural stem cells without affecting hTERT levels (Haik et al., 2000). Other factors known to activate telomerase are epidermal growth factor, estrogen, progesterone, nitric oxide, as well as hypoxia (Kyo et al., 1999; Seimiya et al., 1999; Wang et al., 2000; Vasa et al., 2000; Minamino et al., 2001; Maida et al., 2002).

Conversely to the suggestion that TERT is an anti-apoptotic molecule, TERT expression is suppressed by pro-apoptotic transcription factors such as p53 and Mad-1 (Chin et al., 1999; Li et al., 1999; Oh et al., 2000; Xu et al., 2000). Mad-1 binds similar to c-Myc to the two E-boxes in the hTERT core promoter and seems to markedly suppress hTERT promoter in differentiating cells (Gunes et al., 2000). Other factors known to suppress hTERT and telomerase are methylation of hTERT gene promoter and PTEN (Devereux et al., 1999; Tian et al., 1999).

### 3.2 IMMORTALIZATION OF CELLS

Since hTERT is reported to be the limiting factor of telomerase activity, transfection of cells with hTERT should increase the life span of various cell types. Studies of hTERT-transfected human endothelial cells have reported conflicting results. While one study indicated that expression of hTERT is sufficient for immortalization of human endothelial cells (Yang et al., 1999), another study demonstrated that hTERT alone does not immortalize these cells (O'Hare et al., 2001). Further, while introduction of
hTERT alone into primary human skin fibroblasts, retinal pigment epithelial cells and endothelial cells appears to be adequate to immortalize these cells, primary human skin keratinocytes and mammary epithelial cells require downregulation of p16/pRb together with overexpression of hTERT in order to become immortal (Kiyono et al., 1998; Dickson et al., 2000; Farwell et al., 2000). In addition, several laboratories have combined hTERT with oncogenes (e.g. SV40 large T) for immortalization (Zhu et al., 1999; Thomas et al., 2000; O’Hare et al., 2001). This might suggest a cell type specificity of hTERT-mediated immortalization.

Several studies have reported that immortalization of human primary cells is a rare event. Two recent studies suggested that this was due to inadequate culture conditions (Ramirez et al., 2001; Wright and Shay, 2001). They also proposed that both mammary epithelial cells and keratinocytes can be immortalized with hTERT alone if adequate growth conditions are supplied. However, these observations are controversial and others have reported that even if keratinocytes are continuously grown on feeder layers, inactivation of p16 is required for immortalization (Dickson et al., 2000). It is possible that mammary epithelial cells and keratinocytes immortalized with hTERT alone have spontaneously downregulated their p16 by hypermethylation, since p16 dysfunction can sometimes occur in these cells at very early stages of the immortalization process (Loughran et al., 1996). In addition, endothelial cells are reported to be equally resistant to immortalization with hTERT, whether cultured in ECGM or under the conditions shown by Yang et al. to be adequate for immortalization (Yang et al., 1999; MacKenzie et al., 2002). Furthermore, three different growth patterns for hTERT cells cultured under identical conditions have been observed. These results strongly suggest that culture conditions were not responsible for variations in the proliferative response of endothelial cells transduced with hTERT (MacKenzie et al., 2002). In addition to endothelial cells, MRC-5 foetal lung fibroblasts transduced with hTERT eventually enter a growth crisis and are not universally immortalized (Franco et al., 2001; MacKenzie et al., 2000).

Immortalization of endothelial cells with SV40T together with hTERT appears to be much more efficient than hTERT alone (MacKenzie et al., 2002). In addition to hTERT and SV40T, endothelial cells have been immortalized by HPV-16 E6/E7, murine
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sarcoma virus and Kaposi’s sarcoma associated herpesvirus G-protein coupled receptor (Faller et al., 1988; Lassalle et al., 1992; Bailey et al., 1994; Fontijn et al., 1995; Burger et al., 1998; Matsushita et al., 2001; Minamino et al., 2002; Bais et al., 2003). Several previous studies have demonstrated that SV40T-transfected endothelial cells encounter a growth crisis after an extended period of proliferation, indicating that the telomerase is the limiting factor at crisis. Furthermore, even if telomerase activity is spontaneously upregulated in SV40 or HPV-16 E6/E7 transformed cells, these cells usually do not maintain normal morphology, karyotype or typical endothelial cell antigens (discussed further below). Thus, hTERT transfection-overexpression might be the best way to immortalize cells and the same time retain their characteristic phenotypes.

3.3 IMMORTALIZED CELLS

Despite high levels of telomerase activity, the telomeres in several types of hTERT-immortalized cells continue to shorten as the cells proliferate beyond senescence (Ouellette et al., 1999; Yang et al., 1999; Zhu et al., 1999; Farwell et al., 2000; MacKenzie et al., 2002) This finding is surprising since the key function of TERT is thought to be to maintain and extend the telomeric repeats that are essential for genomic integrity and stability. These observations have led to the proposal of two models in order to explain these findings. The first model suggests that the telomerase lengthens the shortest telomeres under conditions of limiting telomerase activity (Ouellette et al., 2000). According to the second model immortalization by telomerase may further promote cell proliferation by means of a "capping" function, that prevents telomeric fusions (Zhu et al., 1999; Blackburn, 2001).

Endothelial cells transfected with TERT appear to retain their primary endothelial cell phenotype, dependency on ECGF for growth, normal cobblestone morphology, expression of EC-specific antigens and normal EC functions (Yang et al., 1999; MacKenzie et al., 2002). In contrast to hTERT overexpression, SV40T transfection seems to partially transform endothelial cells (MacKenzie et al., 2002). Transfection of human endothelial cells with the SV40 early genes increased their replicative potential, but the cells eventually senesced or developed abnormal morphologies (Fickling et al., 1992; Hohenwarter et al., 1992; Lassalle et al., 1992; Hohenwarter et al., 1997). Although some of the cells retain a normal EC morphology and grow at a normal rate,
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These cells also display an extended lifespan, reduced expression of certain EC antigens like KDR, VWF, P-CAM and CD34, and grow independently of ECGF (Fickling et al., 1992; Hohenwarter et al., 1992; Lassalle et al., 1992; Hohenwarter et al., 1997).

Another characteristic of immortalized cells is their resistance to apoptosis. (Fu et al., 1999b; Holt et al., 1999; Xiang et al., 2000; Ren et al., 2001; Gorbunova et al., 2002). The reason for property in not clear, but TERT appears to modulate the proto-oncogenes c-jun and c-Fos, and downregulates p53, bcl-xs, and caspase-3, but only slightly increases bcl-2 mRNA levels (Xiang et al., 2000). Furthermore, inhibition of telomerase activity increases sensitivity to apoptosis and this can be counteracted with overexpression of Bcl-2, or the caspase inhibitor z-VAD-fmk (Fu et al., 1999b). Suppression of TERT expression in cultured embryonic brain neurons using antisense technology causes neurons to undergo apoptosis (Fu et al., 2000).

Some telomerase expressing cells undergoing apoptosis, or DNA-damage-induced senescence have been reported to demonstrate pronounced telomere shortening (Chen et al., 2001; Ren et al., 2001). One mechanism as to how TERT might promote cell survival is by suppressing DNA damage, or signals generated in response to DNA damage, by stabilizing chromosome ends (Holt et al., 1999). During apoptosis, the shortened telomeres may no longer protect DNA. As a result, damages could accumulate, leading to cellular senescence and apoptosis. Furthermore, it has been suggested that Akt might promote cell survival by phosphorylating TERT and thereby modify its enzymatic activity (Kang et al., 1999). Although TERT is often localized in the nucleus, TERT is also present in the cytoplasma of the cells, where it is believed to modify proteins such as Bcl-2, which are involved in protecting cells from apoptosis (Fu et al., 2000).

3.4 TELOMERASE AND ANGIOGENESIS

The identification of the gene encoding mouse telomerase RNA (mTR) has made it possible to address the importance of telomerase for viability, chromosome stability, and tumorigenesis in mammals. Knockout mice in which the mTR gene is deleted, lack detectable telomerase activity, but are viable for the six generations analyzed (Blasco et al., 1997). Age-dependent telomere shortening and associated genetic instability are
Telomerase associated with a shortened lifespan, as well as a reduced ability to respond to stresses such as wound healing and hematopoietic ablation (Rudolph et al., 1999). Endothelial cells from these mice show decreased angiogenic potential in a matrigel assay. The same mice also reveal a decreased tumor growth rate after implantation of tumor cells, associated with decreased microvessel density in the developing tumors (Pallini et al., 2001). Furthermore, telomerase deficiency and telomere shortening lead to pathological cardiac remodeling and severe ventricular dysfunction, coupled with impaired cardiomyocyte regeneration and apoptosis (Leri et al., 2003). In humans, endothelial cell senescence has been shown to be involved in atherosclerosis, suggesting a role of telomere dysfunction in the pathogenesis of atherosclerotic plaques (Minamino et al., 2002). In a murine hindlimb ischemia model, hTERT-transfected endothelial progenitor cells improved salvage of the ischemic limb and increased neovascularization compared with untransfected control cells (Murasawa et al., 2002). Finally, endothelial cells associated with angiogenesis of human astrocytic tumors show reactivation of telomerase (Pallini et al., 2001).
4 PRESENT WORK

When starting my thesis studies, only a dozen of the angiogenesis inhibitors we know today had been discovered and characterized. Angiostatin was proved to inhibit endothelial proliferation, migration and tube formation. There were no known receptors for angiostatin or knowledge of the underlying mechanisms. Although angiostatin was reported to inhibit neovascularization and tumor growth, the high amounts of angiostatin required for patient treatment urged us to search alternative candidates. The main goals with this thesis have been to identify novel and potent angiogenesis inhibitors and to understand the underlying mechanisms.

More specifically the aims were:

1. To identify and characterize a novel and potent angiogenesis inhibitor, K1-5
2. To investigate the molecular mechanisms underlying the antiangiogenic and anti-tumor properties of K1-5
3. To determine the novel role of adipose-tissue derived adiponectin in angiogenesis
4. To establish a stable and standardized endothelial cell line with good angiogenic properties and examine the role of telomerase in immortalized endothelial cells
4.1 METHODS

Apart from standard molecular biology and cell culture techniques more specialized techniques are listed in the table below. For detailed descriptions, see the materials and methods in their respective referred papers.

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5 RESULTS AND DISCUSSION

5.1 SUPPRESSION OF ANGIOGENESIS AND TUMOR GROWTH BY THE INHIBITOR K1-5 GENERATED BY PLASMIN-MEDIATED PROTEOLYSIS

Angiostatin, an endothelial specific inhibitor, was first isolated from both serum and urine of mouse bearing Lewis lung carcinoma in Dr. Judah Folkman's laboratory (O'Reilly et al., 1994). Angiostatin contains the first four triple disulphide-linked loops of plasminogen known as kringle domains. Shortly after angiostatin was discovered, it was found that kringle 5 of human plasminogen, which is not included in angiostatin, also demonstrates inhibitory activity at least as potently as angiostatin (Cao et al., 1997). Our hypothesis was that a fragment containing both angiostatin (kringle 1-4) and kringle 5 could be more potent than angiostatin alone to inhibit angiogenesis and tumor growth \textit{in vivo}.

The proteolytic human kringle 1-5 (K1-5) fragment was prepared by digestion of purified plasminogen with urokinase-activated plasmin. Purified K1-5 fragment was able to inhibit bFGF-stimulated bovine capillary endothelial (BCE) cell proliferation in a dose-dependent manner. The anti-proliferative effect of K1-5 appeared to be endothelial cell specific since the concentration required for maximal inhibition of BCE cells did not affect several non-endothelial cell lines. To study the \textit{in vivo} antiangiogenic activity, K1-5 was first tested in the chick chorioallantoic membrane (CAM) assay. K1-5 inhibited new embryonic blood vessel growth at a dose of 5-25 microgram/disk, as measured by formation of avascular zones. To further investigate the antiangiogenic activity of K1-5 and to compare the antiangiogenic activity with angiostatin, the inhibitory effect of systemic administration of K1-5 and angiostatin were tested in the mouse corneal micropocket assay. Systemic treatment by subcutaneous K1-5 injections of mice significantly blocked bFGF-induced corneal neovascularization whereas angiostatin, at the same dose, did not have any significant effect.

Since our results indicated that K1-5 is a potent angiogenic inhibitor, we decided to determine the antitumor activity of K1-5. C57BL/6 mice bearing subcutaneously
implanted primary T241 fibrosarcomas were treated with subcutaneous injections either of K1-5 or angiostatin. Systemic treatment with K1-5 suppressed the tumor growth by approximately 63% when compared to the control group treated with PBS. In contrast, angiostatin at this low dose was not able to inhibit tumor growth significantly. Our findings show that at low doses, K1-5 displays a more potent effect on suppression of endothelial cell proliferation, angiogenesis and tumor growth, than angiostatin.

In this paper we have shown that K1-5 is a potent angiogenesis inhibitor, being more potent than angiostatin. This increased potency of K1-5 might be due to longer half-life in the body. Alternatively, kringle 5 might use different endothelial receptors than angiostatin to transduce its inhibitory effects. K5 alone has been shown to be as potent as angiostatin to inhibit BCE proliferation and migration (Cao et al., 1997; Ji et al., 1998a; Lu et al., 1999). Further, K5 alone can induce apoptosis in endothelial cells (Lu et al., 1999). In retinal vascular endothelial cells and pericytes, K5 downregulates endogenous VEGF and upregulates pigment epithelium-derived factor (PEDF) (Gao et al., 2002). The regulation of endogenous angiogenic factors may be responsible in part for its anti-angiogenic activity. In our experiments, angiostatin and kringle 5 synergistically suppressed endothelial cell growth in vitro and K1–5 exhibited comparable inhibitory effect with that of angiostatin plus K5. If K5 utilizes separate signaling pathways than angiostatin, K1-5 might be able to activate both these pathways and thereby show higher potential to inhibit angiogenesis and tumor growth than angiostatin. Indeed, K1–5 significantly reduced tumor growth in mice at a low dose, where angiostatin did not have any significant effect.

Angiostatin is currently in clinical trials for cancer treatment. Patients show good tolerance to angiostatin and no toxicity have been observed. Since K1-5 was shown to have more potent activity than angiostatin at low doses, the immediately question rised was whether K1-5 could inhibit human tumors at lower dosages than angiostatin. However, it should be emphasized that K1–5 did not completely arrest tumor growth in animal model systems and it will most likely not do so in human cancer patients. Rather, K1-5 should be combined with other modalities. Indeed, angiostatin shows higher efficacy in combination with other therapies in animal models and is currently in clinical trials in combination with radiation therapy and chemotherapy (Gorski et al.,
K1-5 reduces significantly, not only the tumor growth rate, but also the severity of murine collagen-induced arthritis (Sumariwalla et al., 2002). Thus, K1-5 possibly will have therapeutic implications in the treatment of various angiogenic diseases, such as rheumatoid arthritis, diabetic retinopathy, atheroclerosis, tumor growth and metastasis.

5.2 ENDOTHELIAL CELL SURFACE ATP SYNTHASE-TRIGGERED CASPASE-APOPTOTIC PATHWAY IS ESSENTIAL FOR K1-5-INDUCED ANTIANGIOGENESIS

Although angiostatin was discovered several years ago, little is known about the principal mechanisms of how this molecule specifically inhibits endothelial cell growth and angiogenesis. The first paper in this thesis reported the identification of kringle 1-5 (K1-5) of plasminogen, an angiostatin-related proteolytic fragment, as a potent and specific inhibitor of angiogenesis and tumor growth. In this second paper we showed that K1-5-induced endothelial apoptosis is essential for its antiangiogenic activity. K1-5-induced apoptosis involves the activation of pro-caspase-3; a common effector caspase in the apoptotic process and this activation was both dose- and time-dependent. The cell permeable irreversible caspase-3 inhibitor, DEVD-fmk, efficiently blocked K1-5-induced apoptosis in BCE cells and antagonized the inhibition of angiogenesis in in vivo assays including the CAM assay and mouse corneal micropocket assay. Further, the caspase-3 inhibitor abolished the anti-tumor activity of K1-5 by protecting the tumor vasculature from apoptosis. These results indicate that the caspase-3-mediated endothelial apoptosis is essential for the angiostatic activity of K1-5. Further analyses revealed that caspase-8 appears to be the activator caspase, followed by activation of caspase-3 and then caspase-9 amplifying caspase-3 activity. It has previously been reported that angiostatin binds to cell-surface F₀F₁-ATP synthase. Antibodies against F₀F₁-ATP synthases partially blocked K1-5-induced apoptosis and ELISA experiments revealed that K1-5 binds to the ATP synthase and inhibit its activity. Our findings demonstrate that K1-5 displays its properties to inhibit angiogenesis by inducing endothelial apoptosis and this induction involves cell surface F₀F₁-ATP synthase. The importance of F₀F₁-ATP synthase (also called ATP synthase) in apoptosis has been
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previously demonstrated. Matsuyama et al. reported that mitochondrial $F_0F_1$-ATP synthase is apparently required for optimal induction of apoptosis and activation of caspases by Bax in mammalian cells (Matsuyama et al., 1998). Later the same group reported that activation of cytosolic caspases by cytochrome C (cytC) *in vitro* seems to be minimal at neutral pH, but maximal at acidic pH, indicating that mitochondria-induced acidification of the cytosol may be important for caspase activation, although the precise mechanism of this sequence of events is still unclear. Cytosol acidification and cytC release was suppressed by oligomycin, a $F_0F_1$-ATP synthase inhibitor indicating its role in this process (Matsuyama et al., 2000). Angiostatin binds to membrane bound $F_0F_1$-ATP synthase, decreases intracellular pH and induces apoptosis or anoikis dependent on the type of endothelial cells (Lucas et al., 1998; Moser et al., 1999; Wahl and Grant, 2000; Wahl et al., 2002). We have shown that K1-5 can bind $F_0F_1$-ATP synthase and that inhibition of this ATP synthase by specific antibodies reduces K1-5-induced apoptosis in BCE cells, indicating an important role of $F_0F_1$-ATP synthase in this process. Furthermore, in this case apoptosis of endothelial cells seems to be the mechanism by which K1-5 inhibits angiogenesis *in vivo*, since treatment with a caspase-3 inhibitor totally blocks K1-5's effect on angiogenesis and tumor growth.

Regulation of ATP synthase-dependent pro-caspase-8 activation remains to be examined. It is possible that pro-caspase-8 binds directly to the ATP synthase or via
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Adapter proteins, such as Fas-associated death domain (FADD) or TNF receptor associated death domain (TRADD). For example, pro-caspase-8 has been demonstrated to bind to to unligated integrins (Stupack et al., 2001). Therefore, it might be possible that pro-caspase-8 binds to ATP synthase as well. Depending on stimuli and type of cells, caspase-8 might activate two different pathways. In so-called type II cells, active caspase-8 cleave BH3-only protein Bid, which in turn induces translocation, oligomerization and insertion of Bax and/or Bak into mitochondria. This is followed by release of cytC and activation of pro-caspase-9 within the apoptosome complex. In type I cells, active caspase-8 cleaves pro-caspase-3, which is followed by activation of an amplification loop, including the sequential activation of pro-caspase-9 and -3. We have shown that caspase-8 activates caspase-3, which in turn can process and activate pro-caspase-9 to amplify the apoptotic signals. This suggests that K1-5-induced apoptosis requires the proper function of the latter pathway. In fact, all this might occur in or nearby caveoli. ATP synthase is located in caveoli and recently it has been reported that caspase-3 can also be located in caveoli (Stupack et al., 2001; Moser et al., 2002).

K1-5 might cause apoptosis by binding to ATP synthase in the cell membrane, decreasing intracellular pH and thereby initiating the activation of the pro-caspases. Alternatively, ATP synthase might signal directly or indirectly to p53. Recently it has been shown that p53 can activate caspase-8 in a transcription-independent manner (Ding et al., 2000). This activation seemed to be independent of Fas-associated death domain protein (FADD), and caspase-8 activation was found in a 600-kDa complex following p53 activation. In support of our hypothesis, angiostatin-induced apoptosis has been reported to be dependent on p53 (Jimenez et al., 2000). In addition, it has previously been documented that inhibition of vacuolar ATPase in cardiomyocytes by bafilomycin A increases expression levels of both p53 and p21, and induces apoptosis probably through a p53-mediated pathway (Long et al., 1998). It remains to be studied if K1-5 can induce p53 and p21 expression in BCE cells and if antibodies against the F1-part of ATP synthase could inhibit this induction.

Angiostatin-related fragments are not the only proteins that bind to ATP synthase. It has been reported that the C-terminal domain of p43, endothelial monocyte-activating polypeptide II (EMAP II), also binds to the α-subunit of ATP synthase and induces
endothelial cell apoptosis (Chang et al., 2002). In addition, it has been reported that EMAP II upregulates expression of TNF receptor-1 (TNFR-1) (Berger et al., 2000). To further complicate the picture, a recent report states that TNFR-1 itself might be an ATPase (Miki and Eddy, 2002). It remains to be seen if there are more angiogenesis inhibitors that utilize the ATP synthase for induction of apoptosis.

Interestingly, the apolipoprotein A-I receptor for hepatic high-density lipoprotein (HDL) was found to be an ATP synthase (Martinez et al., 2003). ApoA-I-binding stimulated endocytosis of holo-HDL and this endocytosis were strictly dependent on ATP hydrolysis. It would be tempting to study if angiostatin or K1-5 can cause endocytosis when bound to ATP synthase on endothelial cells. In addition, ATP synthase was found to have other properties as well. It was shown to be a ligand in the effector phase for natural killer (NK)-cell mediated cytotoxicity (Das et al., 1994).

It is possible that angiostatin binds to and interferes with not only the F_0F_1-ATP synthase, but also other ATPases. For example, Contreras et al. showed that Na^+K^-ATPases are involved in cell attachment. Inhibition of this ATPase led to increases in intracellular calcium concentrations, tyrosine phosphorylation, disturbance in pattern of cell-cell and cell-substrate molecules and cell detachment (Contreras et al., 1999). This could be the case in HUVE cells where angiostatin binding to ECs has been shown to disrupt focal adhesion kinase (FAK) signaling (Claesson-Welsh et al., 1998). Since the Na^+H^-antiporter is located in FAK plaques (Grinstein et al., 1993) angiostatin and K1-5 might impede cell attachment and thereby cause anoikis by interfering Na^+K^- or Na^+H^-ATPases (Wahl et al., 2002).

I have discussed the role of different ATP synthases and ATPases in apoptosis, but the ATPases might not be the only molecules involved. It is possible that several receptors or cell surface proteins act together in order to have an effect on the signaling. For example, inhibition of neovascularization in vivo by thrombospondin-1 is dependent on both CD36 and Fas-signalling (Jimenez et al., 2000; Volpert et al., 2002). Binding of thrombospondin-1 to its receptor CD36 upregulates expression of Fas ligand. This indicates that CD36 might be the receptor for thrombospondin-1, although Fas-signaling is required for effective induction of apoptosis. It is possible that angiostatin and K1-5 require Fas, TNFR or other death receptors together with ATP synthase in
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order to induce apoptosis. Alternatively, angiostatin might block survival signals by
binding to \( \alpha v \beta 3 \) integrins (Tarui et al., 2001) and this, together with ATP synthase,
might be necessary for apoptosis. There might still be some unidentified receptors that
are required for mediating the effect of angiostatin and K1-5. However, according to
our studies, the role of ATP synthase seems to be essential in mediating the apoptotic
signals of K1-5. It remains to be studied if antibodies against ATP synthase can block
the effect of K1-5 on angiogenesis and tumor growth \textit{in vivo}.

5.3 ADIPONECTIN-INDUCED ANTIANGIOGENESIS AND ANTITUMOR
ACTIVITY INVOLVE CASPASE-MEDIATED ENDOTHELIAL
APOPTOSIS

Several laboratories have reported that adipose tissue can regulate angiogenesis. For
example, leptin, a pleiotropic hormone secreted from the adipose tissue, regulates
food intake, metabolic and endocrine responses and plays a regulatory role in
hematopoiesis, inflammation, immunity, and angiogenesis (Zhang et al., 1994;
Gainsford et al., 1996; Bouloumie et al., 1998; Loffreda et al., 1998; Sierra-
Honigmann et al., 1998). Leptin potently induces neovascularization by stimulating
endothelial cell proliferation and cell survival through activation of the endothelial \( \textit{ob} \)
receptor (Bouloumie et al., 1998). We investigated another adipocyte-specific
secretory protein, adiponectin, which has recently been implicated as a mediator of
systemic insulin sensitivity. In \textit{in vitro} assays, adiponectin appeared to be a potent
endothelial cell inhibitor, inhibiting both proliferation and migration of these cells. In
addition to endothelial cells, adiponectin significantly inhibited SMC proliferation,
although at higher concentrations. This novel angiogenesis inhibitor potently
suppressed newly formed blood vessels in the CAM and inhibited bFGF-induced
neovascularization in the mouse cornea assay. Further, adiponectin was able to
suppress tumor growth when T421 fibrosarcoma bearing mice were treated with
intra-lesional injections of adiponectin. Adiponectin-treated tumors had significantly
reduced neovascularization as compared with control tumors, and this reduction of
blood vessels led to increased tumor cell apoptosis. Further experiments demonstrated
that adiponectin, like K1-5, induced apoptosis in BCE cells, and this induction was
caspase-3, -8 and -9-dependent.
Results and discussion

How adiponectin inhibits tumor growth is not clear. It is possible that adiponectin inhibits endothelial growth directly or induces endothelial apoptosis. However, there are several other possibilities to adiponectin's mechanisms. First it is possible that the apoptotic properties of adiponectin are due to upregulation of CD36 (Yamauchi et al., 2001), since thrombospondin-1 has been reported to induce apoptosis via this receptor (Jimenez et al., 2000). Secondly, adiponectin has been shown to have anti-inflammatory properties (Yokota et al., 2000). It is possible that adiponectin regulates inflammatory cell entrance to the tumor and thereby decreases the release of angiogenic factors from these cells. Furthermore, adiponectin has a high affinity to gelatin and might interfere with cellular adhesion (Nakano et al., 1996). Indeed it has been shown that adiponectin attenuates induction of adhesion molecules induced by TNF-α (Ouchi et al., 1999). Alternatively, since adiponectin structurally resembles TNF-α, it might antagonize TNF-α activity (Shapiro and Scherer, 1998). Adiponectin has been reported to inhibit smooth muscle cell proliferation and migration and interfering directly with PDGF-BB, a factor involved in blood vessel maturation (Arita et al., 2002; Matsuda et al., 2002). Finally, since adiponectin is known to regulate insulin sensitivity and glucose clearance, it might affect tumor metabolism (Berg et al., 2001).

Whether adiponectin acts directly on endothelial cells and/or uses other mechanisms, remains to be seen. However, whatever the mechanism, adiponectin appears to potently inhibit tumor growth. Maybe its potency is due to several actions on tumor vessels. It would be interesting to use adiponectin knock-out mice and adiponectin overexpressing mice to study angiogenesis and tumor growth, especially the mice overexpressing adiponectin. Could overexpression of the globular domain of adiponectin inhibit angiogenesis in in vivo assays, such as the corneal assay and wound healing, or reduce tumor growth? At least it is known that adiponectin overexpression protected apoE deficient mice from atherosclerosis (Yamauchi et al., 2003), a disease well known to be dependent on angiogenesis. Whether adiponectin can inhibit angiogenesis in other diseases remains to be examined.

Even though no receptor has yet been identified for adiponectin, one possible receptor has been proposed (Yokota et al., 2000). Adiponectin binds to C1qRp, a receptor for complement factor C1q, which might modulate phagocytic activity by adiponectin.
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(Yokota et al., 2000). However, the definitive role of this binding is not known. The close structural similarities between adiponectin and TNF-α suggest that the receptor for TNF-α might be one of adiponectin's targets (Shapiro and Scherer, 1998). However adiponectin does not affect TNF-α binding to TNF receptor although it specifically inhibits the IkB-α–NF-κB pathway stimulated by TNF-α (Ouchi et al., 2000). TNF-α is a general inducer of cellular apoptosis, usually when the transcriptional inhibitor cyclohexamine is added. However, adiponectin is able to induce apoptosis without addition of cyclohexamine, which might suggest that apoptosis induced by TNF-α or adiponectin are not entirely identical. It is possible that adiponectin binds to other death receptors in the TNFR family, or we may find a novel receptor for adiponectin. However, since adiponectin appears to have several different functions, it is possible that adiponectin mediates its effects through several receptors, like K1-5 and angiostatin. To identify and characterize these receptors is probably enough study for one or several doctoral theses.

5.4 IMMORTALIZATION OF BOVINE CAPILLARY ENDOTHELIAL CELLS BY HTERT ALONE INVOLVES INACTIVATION OF P16INK4A

When testing K1-5 and adiponectin in in vitro models, we encountered a problem of endothelial cell culture due to the fact that primary capillary endothelial cells can only be cultured for very limited passages. This limitation causes difficulties to standardize the in vitro assays such as endothelial proliferation assay, migration assay and tube formation assay. hTERT (human telomerase reverse transcriptase) have been reported by several laboratories to be able to immortalizes different kinds of cells without any indications of transformation (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Morales et al., 1999). We used the primary pre-senescence BCE cells to transfect with hTERT gene and the positive cells were selected with Hygromycin B.

When senescent primary BCE cells were compared with hTERT immortalized BCE cells (hTERT⁺-BCE) several differences could be observed in their morphology, such as staining ability with senescence-associated β-galactosidase (SA-β-gal), changes in cell size, changes in ratio of cytoplasm/nucleus, and rearrangements of actin filament. To prove that hTERT⁺-BCE do express telomerase, a telomere (TRAP DNA ladder)
Results and discussion

assay was performed. Analysis confirmed that a high level of telomerase activity was
detected in hTERT⁺-BCE cells, whereas primary BCE cells did not show any
detectable telomerase activity. To investigate that hTERT⁺-BCE cells were not
transformed, the cells were subcutaneously implanted into immunodeficient SCID
mice. The hTERT⁺-BCE cells were not able to form tumors in SCID mice, and also the
cells responded as well as the primary cells to both angiogenic factors and inhibitors on
the endothelial proliferation assay, if not better. This might be due to the fact that
transfected cells resemble more the young primary cells and might be more sensitive
for angiogenic signals. The same observation was true for migration assay.

Interestingly we found that the telomere lengths in the hTERT-transfected cells were
shorter than in primary pre-senescent cells. If the telomere length is critical for
initiating senescence and introduction of telomerase can immortalize the cells without
increasing the telomere length, what other role could telomerase play in
immortalization? The fact that telomere lengths were not the critical factor for BCE
cells bypassing replicative senescence led us to hypothesize that immortalization of
these bovine endothelial cells must be mediated by alternative mechanisms.

Several recent studies suggest that the p16/pRb and p21 pathways are involved in
replicative senescence. We investigated if transfection of hTERT into BCE cells could
alter the expression or function of these genes. Since inactivation of p16 is required for
escape from senescence we wanted to test if overexpression of p16 or p15 in
immortalized BCE cells would drive these cells back to senescence. To test this
hypothesis we transducted the hTERT⁺-BCE cells with adenovirus expressing p15 or
p16. To determinate how the p16 is inactivated in immortalized BCE cells we
hypothesized that methylation of these genes could be involved. To test this, hTERT⁺-
BCE cells were treated with a demethylation agent. The treatment was able to
upregulate both p16 and p21 expression and lead the cells to a senescent phenotype.
Since methylation appeared to downregulate p16 expression, we examined expression
of a specific methyltransferase, DNMT-1, and found that the expression was increased
in hTERT⁺-BCE cells. Even though it has been reported that DNMT-1 expression is
regulated by the status of the cell cycle, increased proliferation of hTERT⁺-BCE cell
can not explain the increased levels, since the levels are even higher than in young
actively dividing BCE cells (Szyf et al., 1991).
The hTERT-immortalized BCE cells maintained the same biological properties as non-immortalized BCE cells. This finding is essential for endothelial cell biology research because primary endothelial cells usually become senescent within only very limited passages. For example, human endothelial cells, such as human dermal microvascular endothelial cells, can only be cultured for a few passages in vitro before they enter the senescence. Thus, this short cell culture period makes it more difficult to discover novel endothelial cell stimulators and inhibitors.

In humans, endothelial cell senescence has been shown to be involved in atherosclerosis, suggesting a role of telomere dysfunction in pathogenesis of atherosclerotic plaques (Minamino et al., 2002). In mice, telomerase deficiency and telomere shortening leads to pathological cardiac remodelling and severe ventricular dysfunction coupled with impaired cardiomyocyte regeneration and apoptosis (Leri et al., 2003). Furthermore, these mice show reduced wound healing (Rudolph et al., 1999). Since telomere shortening and senescence appears to be involved in pathogenesis of some diseases, one might wonder if hTERT-transfected cells would improve the state of these diseases. Indeed, hTERT-transfected endothelial cells can function in vivo and form tubes and active circulation (Yang et al., 2001). In a murine hindlimb ischemia model, hTERT-transfected endothelial progenitor cells improve salvage of the ischemic limb and increased neovascularization compared with untransfected control cells (Murasawa et al., 2002). Bovine adrenocortical cells with hTERT form functional tissue and secrete cortisol (Thomas et al., 2000).

Even though TERT does not seem to cause transformation of the cells, a word of causation should be mentioned here. The shortening of the telomeres is considered as a protective mechanism against cancer. Telomerase activity might give a growth advantage to the cells and resistance to apoptosis. Although the risk of mutation is low, do we dare to take that risk? Benefits and risks should be carefully balanced. TERT-cells might increase the length and quality of our lives, but who would like to live forever?
6 CONCLUDING REMARKS

The ultimate goal in anti-cancer therapy is to eliminate cancer entirely from the body. Several anti-angiogenic proteins have been applied in cancer therapy although these candidates do not yet have the ability to suppress tumor growth entirely. Other disadvantages of antiangiogenic protein therapy include repeated injections, prolonged treatment, potential transmission of toxins and other infectious particles, and high cost for manufacturing large amounts of proteins. Thus alternative approaches are required to be developed in order to improve the antiangiogenic therapy. These are identification of new more potent angiogenesis inhibitors, improvement of half-life in circulation, and optimal methods and timing of administration. A combination of antiangiogenic therapy and cytotoxic, radio-, anti-hormonal, or immunotherapy may become more effective because the combined treatment is directed to different compartments. In fact, some reports are already showing encouraging results when anti-angiogenic treatment is combined with other modalities (Gorski et al., 1998; Mauceri et al., 1998; Gyorffy et al., 2001; Sun et al., 2001; Mauceri et al., 2002; te Velde et al., 2002). In addition, the anti-angiogenic compounds might “normalize” the blood flow in the tumors by decreasing resistance due to tortousity and irregular vessel diameter, and thereby increase admission of other therapeutic compounds to the tumor site. Another way to increase treatment efficacy is to target the inhibitor to the tumor tissue. Previous works from several laboratories have shown that when specific short peptides are attached to a reporter protein, the protein specifically resided in various tissues (Pasqualini et al., 1997; Arap et al., 1998; Arap et al., 2002). To fuse these peptides with anti-angiogenic inhibitors could in theory reduce the dosages required for cancer therapy, since the inhibitors would not circulate in the body, but rather target to the tumor area.

The field of angiogenesis literally exploded in the middle of the 1990s with the discovery of several endothelial-specific inhibitors. We are gaining more and more understanding and knowledge of how these molecules work. Even though this thesis has brought some light in regards to understanding the underlying mechanism of a couple of these inhibitors, the work is far from finished. We are just in the beginning of understanding…
7 TIIVISTELMÄ SUOMEKSI/ SUMMARY IN FINNISH


Angiostatiinin on osoitettu estävän syövän kasvua ja etäispesäkkeiden leviämistä hiirillä. Kringle 1-5 (K1-5) on isompia kappale plasminogeeniä kuin angiostatiini ja suorittamamme kokeet ovat osoittaneet että K1-5 ehkäisee endoteelisolujen jakautumisen, uusien verisuonien kasvun ja hidastaa syöpäkasvainten kasvua hiirillä paremmin kuin sen lyhyempi muoto angiostatiini. K1-5 ja angiostatiini tarttuvat tiettyihin molekyyleihin endoteelisoluissa, kuten ATP syntaasiin, ja voi näin altistaa endoteelisolut ohjelmoinnukseen eli apoptoosiin. K1-5 voin näin ohjailta syöpäsolujen kasvua, sillä hapen puute edistää syöpäsolujen kuolemaa.

Toinen angiogeneesin estäjä on adiponectin. Tämä molekyli on tavallisesti alentunut verenkierrossa potilailla, jotka kärsivät ylipainosta, sokeritaudista tai sydäntaudista. Adiponectin altistaa solut insulinin vaikutukselle ja nostaa rasvan hapettumista lihaksissa. Sen on osoitettu myös ehkäisevän arterioskleroosia ja sydäninfarkteja. Me olemme ensimmäistä kertaa osoittaneet että adiponectin voi myös säädellä verisuonien kasvua. Tämä tieto voidaan hyödyntää siten että sokeritaustisten potilaiden tilannetta voidaan helpottaa, ei vain lisäämällä insuliinin herkkyyttä, mutta myös estämällä sokeritaudista johtuvaa verisuonien kasvua silmän verkkokalvossa ja tästä kehittyvää sokeutta.

Verisuonien rappeutuminen on osaksi osoitettu johtuvan endoteelisolujen vanhentumisesta ja niiden jakautumiskyvyn menettämisestä. Koska TERT-solut käyttävät normaaleja ja jakaantuvat rajattomasti, niitä voitaisiin periaatteessa käyttää terapeuttisesti ehkäisemään verisuonien rappeutumisen. Vaihtoehtoisesti, TERT-endoteelisoluja voisi käyttää sellaisissa sairauksissa, joissa tarvitaan uusia verisuonia, kuten aivoverenvuodon ja iskemian terapeuttisessa hoidossa.
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